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EXHIBIT 1

## Vitronectin Receptor Antibodies Inhibit Infection of HeLa and A549 Cells by Adenovirus Type 12 but Not by Adenovirus Type 2

MEI BAI, LAUREN CAMPISI, AND PAUL FREIMUTH\*

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

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The penton base gene from adenovirus type 12 (Ad12) was sequenced and encodes a 497-residue polypeptide, 74 residues shorter than the penton base from Ad2. The Ad2 and Ad12 proteins are highly conserved at the amino- and carboxy-terminal ends but diverge radically in the central region, where 63 residues are missing from the Ad12 sequence. Conserved within this variable region is the sequence Arg-Gly-Asp (RGD), which, in the Ad2 penton base, binds to integrins in the target cell membrane, enhancing the rate or the efficiency of infection. The Ad12 penton base was expressed in *Escherichia coli*, and the purified refolded protein assembled in vitro with Ad2 fibers. In contrast to the Ad2 penton base, the Ad12 protein failed to cause the rounding of adherent cells or to promote attachment of HeLa S3 suspension cells; however, A549 cells did attach to surfaces coated with either protein and pretreatment of the cells with an integrin  $\alpha v \beta 5$  monoclonal antibody reduced attachment to background levels. Treatment of HeLa and A549 cells with integrin  $\alpha v \beta 3$  or  $\alpha v \beta 5$  monoclonal antibodies or with an RGD-containing fragment of the Ad2 penton base protein inhibited infection by Ad12 but had no effect on and in some cases enhanced infection by Ad2. Purified Ad2 fiber protein reduced the binding of radiolabeled Ad2 and Ad12 virions to HeLa and A549 cells nearly to background levels, but the concentrations of fiber that strongly inhibited infection by Ad2 only weakly inhibited Ad12 infection. These data suggest that  $\alpha v$ -containing integrins alone may be sufficient to support infection by Ad12 and that this pathway is not efficiently used by Ad2.

The interaction of adenovirus type 2 (Ad2) with the cell membrane is complex and involves at least two distinct receptors. Earlier reports showed the existence of a cell receptor for the viral fiber subunits (15, 19, 21), which are elongated proteins that project from each of the 12 capsid vertices (20); the receptor ligand resides within a globular structure at the distal end of the fiber molecule (16). The interaction of virus with the fiber receptor accounts for the bulk of virus adsorption to cultured cells since the treatment of cells with purified fiber protein quantitatively blocks the subsequent attachment of virions (15, 21, 38). The identity of this receptor is unknown, although several candidate cellular proteins have been isolated by fiber affinity methods (4, 12, 33).

Fibers are assembled onto the viral penton base capsomers (6, 17), which are pentamers of identical 63-kDa subunits (34) and form the vertices of the icosahedral capsid. An RGD (arginine-glycine-aspartic acid) sequence in the Ad2 penton base (residues 340 to 342) has recently been found to interact with vitronectin-binding integrins in the cell plasma membrane (3, 4, 38). These integrins mediate cell adhesion by binding to RGD sequences in the extracellular matrix (13, 26); exposure of adherent cells to native (10, 18, 25, 35) or recombinant (3) Ad2 penton base protein displaces integrins from the extracellular matrix and ultimately detaches the cells. Ad2 mutants lacking functional penton base RGD sequences were as infectious as wild-type virus but were delayed at an early stage of infection on adherent cells but not on suspension cells (3). The loss of the viral RGD ligand does not appear to be equivalent to the loss of  $\alpha v$ -integrin function, since a mutant cell line that lacks functional vitronectin receptors is partially resistant to infection (38) and vitronectin receptor antibodies and RGD-

containing peptides render integrin-bearing cells partially resistant to infection (4, 38).

Although the infection of cultured cells by Ad2 RGD mutants appears to be normal under certain conditions, the fact that the wild-type virus contains a functional RGD ligand suggests that the interaction between Ad2 and integrins may be an important step in the infection of human cells in vivo. Not all adenovirus serotypes contain an RGD sequence in their penton base subunits; therefore, the interaction with integrins may not be universal. The DNA sequences of the penton base genes from human enteric Ad40 (8, 22) and fowl adenovirus type 10 (FAV-10) (27) were reported earlier. Alignment of the Ad2, Ad40, and FAV-10 sequences shows that the amino- and carboxy-terminal ends of the penton base are highly conserved, but the central region, which contains the RGD sequence in Ad2, in Ad40 and FAV-10 is over 60 residues shorter and lacks an intact RGD sequence (3). Here we report the DNA sequence of the penton base gene from human Ad12 and characterize the interactions of the Ad12 fiber and penton base with their cognate receptors. As with Ad40 and FAV-10, the central region of the Ad12 penton base is over 60 residues shorter than that in Ad2; however, the RGD sequence is conserved. The treatment of HeLa and A549 cells with vitronectin receptor antibodies inhibited infection by Ad12 but had no effect on and in some cases enhanced the efficiency of infection by Ad2. Fiber protein from Ad2 completely blocked the adsorption of radiolabeled Ad2 and Ad12 virions but was a relatively weak inhibitor of Ad12 infection. These data suggest that the vitronectin receptor alone can mediate Ad12 binding and infection and that this pathway is not efficiently used by Ad2. Different possible roles for the vitronectin receptor in Ad2 and Ad12 infections are discussed.

\* Corresponding author. Phone: (516) 282-3350. Fax: (516) 282-3407.

## MATERIALS AND METHODS

**Cells and virus.** A549 cells (ATCC CCL 185) and adherent HeLa cells were grown in Dulbecco's minimal essential medium (DME) supplemented with 10% bovine calf serum (CS) and antibiotics (100 U of penicillin and 100 µg of streptomycin per ml). HeLa S3 cells were grown in suspension in F13 medium supplemented with 10% CS. Ad2 and Ad12 (strain Huie) virus stocks were obtained from C. Anderson, Brookhaven National Laboratory. Virus titers on A549 cells were determined by indirect immunoassay 48 h after infection as described previously (3), with rabbit anti-Ad5 virion serum (obtained from H. S. Ginsberg, Columbia University) to stain cells infected by Ad12 virus and with rabbit anti-Ad2 hexon serum (from C. Anderson, Brookhaven National Laboratory) to stain cells infected by wild-type or mutant Ad2 virus. Primary antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel) and metal-enhanced diaminobenzidine developer as described previously (11). To prepare radiolabeled virus, A549 cells in 10-cm-diameter dishes were infected with 10 focus-forming units per cell and then exposed 16 h later to 0.2 mCi of [<sup>3</sup>H]thymidine per dish. At 48 h after infection, virus was harvested, banded twice in CsCl gradients, and stored in small aliquots at -70°C in 10 mM Tris buffer (pH 7.5) containing 1 mM MgCl<sub>2</sub> and 10% glycerol.

**DNA sequencing.** The *Bam*HI-D fragment of the Ad12 genome (coordinates 34 to 47) was digested at a unique *Hind*III site at coordinate 43, and the two pieces were cloned in pBluescript. Unidirectional deletions from the *Hind*III site in both clones were constructed by digestion with exonuclease III and mung bean nuclease as described previously (39), and the DNA sequence was determined by the chain termination method with vector-specific primers. The sequences of the opposing DNA strands were determined from subcloned restriction fragments. The 1,642-bp DNA sequence we obtained was identical to nucleotides 13291 to 14932 of the complete Ad12 genome sequence reported recently (29).

**Protein expression and purification.** The penton base gene was amplified from the Ad12 genome by PCR with a sense-strand primer, 5'-ACATATGAGGCGCGCGGT, that contained the translation initiator ATG and incorporated an upstream *Nde*I site and with an antisense-strand primer, 5'-TGGATCCGGACATGTTACCACT, that annealed downstream of the translation terminator and introduced a *Bam*HI site. The fragment was cloned by using the TA cloning system (Invitrogen) and then was subsequently cloned between the *Nde*I and *Bam*HI sites of the phage T7-based bacterial expression vector pET11a (31). The penton base was purified from induced cultures of host strain DE3 as described previously (3).

The construction of pET11aPB, the Ad2 penton base gene cloned behind the T7 promoter in vector pET11a, was described previously (3). An amino-terminal fragment of the Ad2 penton base was expressed by introducing a stop codon into pET11aPB after codon 301. This derivative was constructed by oligonucleotide-directed mutagenesis of the subcloned *Hind*III-*Bam*HI fragment as described previously (3, 14) with the mutagenic oligonucleotide 5'-AGATGACACCGACTAGT GAGGGATGGCG, which also converted Glu-301 to Asp. DNA fragments that encoded the poorly conserved central region and the highly conserved carboxy-terminal region of the Ad2 penton base were amplified by PCR and cloned in pET11a. Ad2 DNA from nucleotides 15016 to 15322, encoding penton base residues 290 to 390, was amplified with the sense-strand primer 5'-TAGCCATATGGACGCCTACCAAG

GCAAGC, which converted Val-289 to Met and provided an *Nde*I cloning site, and the reverse primer 5'-ACCGGGATC CTTAACTGCGTTTCTTGCTGTC, which included a *Bam*HI cloning site. The Ad2 DNA fragment from nucleotides 15318 to 15866, encoding a carboxy-terminal fragment of the penton base (residues 390 to 571), was amplified with the sense-strand primer 5'-TAGCCATATGAGTTACAACCTAATAAGCAA, which included an ATG codon and an *Nde*I cloning site, and the reverse primer 5'-CCGGAGATCTTCAAAAAGTGCG GCTCGATA, which included a *Bgl*II cloning site. Both fragments were cloned between the *Nde*I and *Bam*HI sites of vector pET11a and were expressed in host strain DE3.

The Ad2 penton base central fragment was purified by NaCl elution from a DEAE-Sephadex column equilibrated in 50 mM phosphate buffer (pH 6.8). Gel filtration analysis employed a TSK250 preparative column and was run at 2 ml/min in 25 mM MOPS [3-(*N*-morpholino)-propanesulfonic acid] buffer (pH 6.8) containing 150 mM NaCl. The column was calibrated with a mixture of thyroglobulin (670 kDa), bovine serum albumin (BSA) dimers (132 kDa), BSA monomers (66 kDa), ovalbumin (43 kDa), and myoglobin (17 kDa). The concentration of the central fragment stock solution used for all assays was 0.7 mg/ml and was determined by amino acid analysis.

**Penton assembly and nondenaturing gel electrophoresis.** Bacterial inclusion bodies containing either the Ad2 or Ad12 penton base were dissolved in 6 M urea, and proteins were renatured as described previously (3). Proteins were further purified by chromatography on columns of DEAE-Sephadex A50 equilibrated in 50 mM phosphate buffer (pH 7.5) and eluted with linear gradients of NaCl from 0 to 0.3 M. Native hexon, penton, and fiber subunits were purified from Ad2-infected HeLa S3 cells as described previously (7). For fiber assembly experiments, 15 to 18 µg of the Ad2 or Ad12 penton base was mixed with 10 µg of purified Ad2 fiber in a total volume of 70 µl and incubated for 10 min at room temperature. Samples were electrophoresed under nondenaturing conditions in a 1.5-mm-thick linear gradient (4 to 12%) polyacrylamide slab gel with a 3% stacking gel. The gel was run at 45 V for 16 h at room temperature in 50 mM Tris-384 mM glycine (pH 8.5) running buffer. Molecular mass markers, thyroglobulin (669 kDa) and catalase (232 kDa), were obtained from Sigma. Penton base protein concentrations were calculated from the *A*<sub>280</sub> of solutions by using 1.79 optical density units per mg, which was determined by amino acid analysis of the Ad2 protein. Concentrations of fiber and hexon were estimated from the *A*<sub>280</sub> of solutions by using 0.7 optical density units per mg.

**Cell adhesion assays.** Solutions of recombinant Ad2 and Ad12 penton base proteins in 6 M urea were adsorbed to polystyrene wells (Immulon-2; Dynatech) and were assayed for cell adhesion activity as described previously (3). The concentration of protein adsorbed to wells was critical for observation of inhibition of cell binding. Serial 1:3 dilutions of proteins in 6 M urea were adsorbed to polystyrene wells and assayed for the adhesion of A549 cells. The most dilute solution that gave maximal cell binding and a fivefold-more-concentrated solution were used to coat wells for inhibition studies. A549 cells were detached from confluent plates with 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] in phosphate-buffered saline (PBS) and were washed and resuspended in DME-2% CS before being plated. After a 1-h incubation at 37°C, plates were flooded twice with PBS to shake off unattached cells and the remaining adherent cells were fixed in 4% paraformaldehyde dissolved in PBS and were stained with 1% toluidine blue in the same solution. Plates

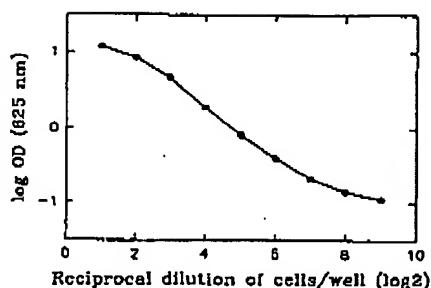


FIG. 1. Quantitation of cell number by toluidine blue staining. Serial 1:2 dilutions of A549 cells were bound to duplicate penton base-coated wells, fixed, stained, and read as described in Materials and Methods. OD, optical density.

were destained with distilled water and air dried. Residual stain was extracted with 200  $\mu$ l of methanol per well, and the  $A_{625}$  was determined with a Cary 1 spectrophotometer. Figure 1 shows a linear relationship between absorbance and cell number within the range of values reported in this paper.

For binding inhibition studies, A549 cells ( $2.5 \times 10^7$ /ml) were maintained in suspension for 1 h at 4°C in DME-2% CS containing antibodies, peptides, or protein inhibitors at the indicated concentrations, diluted to  $10^6$ /ml in DME-2% CS, and plated (50  $\mu$ l per well) on penton base-coated wells. The monoclonal antibody P1F6 (mouse ascites fluid) and synthetic peptides GRGDSP and GRGESF were obtained from Gibco-BRL. P1F6 was shown to bind specifically to the integrin- $\alpha 5\beta 5$  complex (37) by assays similar to those described for clone P5H9 (36). The synthetic peptides were dissolved in PBS at 10 mg/ml and adjusted to neutral pH with 0.1 N NaOH before their addition to cells. Immunoglobulin G from normal rabbit serum was purified on protein G columns as described previously (11), dialyzed against PBS, and adjusted to an  $A_{280}$  of 2.

**Infectivity and virus-binding assays.** A549 and HeLa cells were dissociated from confluent dishes with 5 mM EGTA, washed in Hanks' balanced salt solution containing 2% CS, and resuspended to  $2 \times 10^7$ /ml in Hanks' balanced salt solution-2% CS. Cells were treated in suspension with inhibitors for 1 h on ice and then distributed to BSA-coated flat-bottom 96-well plates and infected in triplicate. After adsorption on ice for 1 h with frequent shaking, warm medium was added and the plates were incubated at 37°C for 30 min prior to being plated on poly-D-lysine (75 to 150 kDa) (Sigma)-coated 24-well cluster plates (Costar). BSA-coated 96-well plates were examined microscopically to confirm that cell transfer was complete. The fiber protein stock solution had an  $A_{280}$  of 0.72. P1F6 (Gibco-BRL) and LM609 (Chemicon) monoclonal antibodies were used in the form of mouse ascites fluid directly as provided by the suppliers.

Ad12 titers were determined on both HeLa S3 and A549 cells as follows. A549 cells were detached from plates by treatment with 5 mM EGTA in PBS, and HeLa S3 cells were centrifuged from F13 medium. Both types of cells were washed and resuspended at  $2 \times 10^7$ /ml in DME-2% CS. Twenty-five microliters of cell suspension was added to 50  $\mu$ l of each virus dilution (in DME-2% CS). After a 1-h incubation at room temperature with frequent agitation to resuspend cells, 0.5 ml of medium was added (F13 was added to HeLa S3 cells) and tubes were centrifuged 7 s in a microcentrifuge. Cells were washed a second time with 0.5 ml of medium, resuspended, and divided equally among 4 wells of a 24-well plate containing

0.5 ml of medium (F13-2% CS for HeLa S3 cells). At 48 h after infection at 37°C, 150  $\mu$ l of cell suspension from each well containing HeLa S3 cells was centrifuged in a cytocentrifuge (Shandon). Slides were air dried, fixed in 100% methanol, and processed for indirect immunofluorescence. The loss of cells from slides during staining procedures was minimal. The number of infected HeLa S3 cells on each slide was multiplied by 3.3 to determine the total number of infected cells per well. A549 cells attached to the wells and were processed for indirect immunofluorescence.

For virus-binding assays, cells were detached and treated with competitors as described above prior to the addition of  $10^5$  [ $^3$ H]thymidine-labeled virions per cell. After 1 h at 4°C, each virus-cell suspension was layered atop 3 ml of PBS containing 10% sucrose and 2% BSA and was centrifuged in a swinging bucket rotor. Pellets were dissolved in 100  $\mu$ l of 0.3 N NaOH, and cell-associated radioactivity was measured by liquid scintillation counting.

## RESULTS

**Sequence of the Ad12 penton base gene and expression in *Escherichia coli*.** The Ad2 penton base gene is located between

Ad12 (1)	MRAVELQTVAPPETPPPSYETVMAAP-----PTVPPRY
Ad2 (1)	MQR-----AHYEGPPPTSEGVSAAPVAALGGSPDAPLDPFPVFFRY
Ad12 (36)	LQPTGRNRLRYSELSPLVDTRVYLVDNRSSDIASLNVONDHNFLLTV
Ad2 (46)	LAPTGRNRLRYSELSPLVDTRVYLVDNRSSDIASLNVONDHNFLLTV
Ad12 (86)	VQNDYSPTEACTQTINFDERSRWGLDKLTILNTHMFWNDHNFLLTV
Ad2 (96)	IQNDYSPTEACTQTINFDERSRWGLDKLTILNTHMFWNDHNFLLTV
Ad12 (136)	RVMARKTNDQQTILSYEASVFLPEQNYSETHITDLAGNATIEHYLAV
Ad2 (146)	RVMRSRLTKQKVELAYENVEPTLPENYSETHITDLAGNATIEHYLAV
Ad12 (186)	GRQGVLESDIGVRFDTNRFGLDFPTQLVTPGVYTFEAFHPDILLPG
Ad2 (196)	GRQGVLESDIGVRFDTNRFGLDFPTQLVTPGVYTFEAFHPDILLPG
Ad12 (236)	CGVDFTESRLNGLIKRQFFQGFQVIMYHELEGGNIPALLQVKKYENS
Ad2 (246)	CGVDFTESRLNGLIKRQFFQGFQVIMYHELEGGNIPALLQVKKYENS
Ad12 (286)	LQDQNT-----VRGDNF-
Ad2 (296)	LQDQNT-----VRGDNF-
Ad12 (312)	-----IALNKAAR-----TEPVETDPKGRSYNLLP
Ad2 (346)	TRAEKRAEAEAAAEAAAPAEQVEKPKKPVTKPLTEDSKKRSYGLIS
Ad12 (323)	-DSQRTKYSWYLAIFYGDFPKGVRSWILLTTPDVTGCGSEQVYSELFDNY
Ad2 (396)	NDSTFTQTRSWYLAIFYGDFPKGVRSWILLTTPDVTGCGSEQVYSELFDNY
Ad12 (372)	QDMVTFRRSRQVSNYPVVAELLPVHAKFTNEDQAVYSQLIRQSTALRV
Ad2 (446)	QDMVTFRRSRQVSNYPVVAELLPVHAKFTNEDQAVYSQLIRQSTALRV
Ad12 (422)	FNRFPENQILVRFAATITTVSENVFALTDGCTPLRSGISGVQVITTD
Ad2 (496)	FNRFPENQILVRFAATITTVSENVFALTDGCTPLRSGISGVQVITTD
Ad12 (472)	ARRATCTVYKALGIVSFRVLSERTP
Ad2 (546)	ARRATCTVYKALGIVSFRVLSERTP

FIG. 2. Alignment of Ad2 and Ad12 penton base protein sequences. Sequences were aligned manually. Colons mark the positions of identical residues, and each dash indicates the absence of a residue at that position. Residue numbers are shown in parentheses.

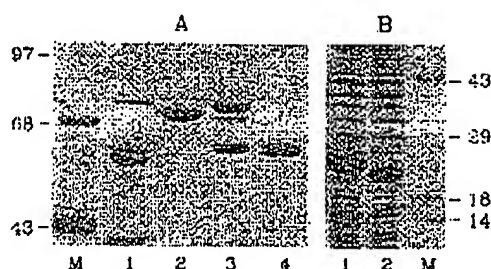


FIG. 3. Electrophoresis of Ad2 and Ad12 penton base proteins on SDS-polyacrylamide gels. (A) Comparison of the apparent molecular masses of the intact recombinant Ad12 penton base in a lysate of induced bacterial cells (lane 1), purified recombinant Ad2 penton base (lane 2), native Ad2 penton base-fiber complexes (lane 3), and native Ad2 fiber (lane 4). (B) Whole-cell lysates of bacteria expressing fragments of the Ad2 penton base extending from residues 390 to 571 (lane 1) and 290 to 390 (lane 2). The molecular masses (in kilodaltons) of protein standards run in lanes M are indicated.

coordinates 39 and 44 of the viral genome (24). To determine whether the Ad12 penton base gene had the same relative position, restriction fragments derived from this region of the Ad12 genome (32) were cloned and their DNA sequences were partially determined. Sequencing in both directions from a *HindIII* site at coordinate 43 revealed an open reading frame that encoded a protein homologous to the Ad2 penton base centered about serine 474. Sequences of DNA fragments subcloned from the Ad12 *Bam*HI-D fragment (coordinates 34 to 47) were assembled to obtain the complete gene sequence. The 1,642-bp sequence we obtained (results not shown) was identical to nucleotides 13291 to 14932 of the complete Ad12 genome sequence recently reported (29). Translation initiation at the first ATG in the 1,563-bp open reading frame would produce a 497-residue polypeptide, 74 residues shorter than the Ad2 penton base. The variation in polypeptide chain length was not randomly distributed across the molecule but occurred predominantly in the central region of the protein through deletions (relative to Ad2) of 37, 15, and 11 residues. In addition, one deletion of 15 residues occurred near the amino-terminal end, as shown in Fig. 2.

The penton base gene was amplified from the Ad12 genome by PCR and cloned in the bacteriophage T7-based expression vector pET11a (31). The expressed protein had an apparent molecular mass of 60 kDa in sodium dodecyl sulfate (SDS) gels (Fig. 3A, lane 1), close to its predicted value of 56.3 kDa. This was in contrast to the recombinant Ad2 penton base, which had an apparent molecular mass of 73 kDa (Fig. 3A, lane 2), about 10 kDa greater than its predicted value of 63.3 kDa. Lanes 3 and 4 of Fig. 3A contained native penton base-fiber complexes and uncomplexed fiber, respectively, purified from Ad2-infected cells. The majority of the native penton base had slower mobility than the recombinant protein, probably reflecting posttranslational modification. The nature of this modification is unknown, although the treatment of native Ad2 penton base with alkaline phosphatase failed to alter its mobility in SDS-polyacrylamide gels (results not shown), and the penton base in virus prepared from cells grown in  $^{32}\text{P}$  was not labeled (1). Most preparations of the native Ad2 penton base contained a small amount of a faster-migrating species that nearly comigrated with the recombinant protein, as seen in lane 3 of Fig. 3A.

These results suggest that the residues in the central region of the Ad2 penton base that are missing in the Ad12 protein

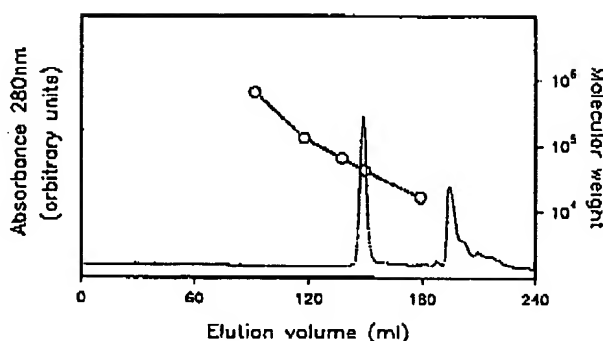


FIG. 4. Gel filtration of the Ad2 penton base central fragment. Two milliliters (20 mg) of purified Ad2 penton base central fragment was injected onto a TSK250 gel filtration column equilibrated in 25 mM MOPS buffer containing 150 mM NaCl. The chromatogram was developed at 2 ml/min (solid line). The peak elution volumes of calibration standards (open circles) are shown and connected with a dashed line.

retard the mobility of the intact protein in SDS-polyacrylamide gels, a conclusion supported by the observation that the poorly conserved central region expressed independently also had an apparent molecular mass substantially greater than the expected value. The Ad2 penton base gene was subcloned in three separate fragments whose endpoints correspond to the boundaries between highly conserved and poorly conserved regions (residues 1 to 301, 290 to 390, and 390 to 571) (Fig. 2). Each fragment was expressed in bacteria, and the polypeptides were examined by electrophoresis in SDS gels. Both the amino-terminal fragment (data not shown) and the carboxy-terminal fragment (Fig. 3B, lane 1) had apparent molecular masses close to the predicted values (33.8 [predicted] and 36 kDa [apparent] for the amino-terminal fragment and 20.6 [predicted] and 23 kDa [apparent] for the carboxy-terminal fragment). The central fragment (Fig. 3B, lane 2) had an apparent molecular mass of 21 kDa, about 10 kDa greater than the predicted value of 10.4 kDa. The central fragment eluted from gel filtration columns as a 43-kDa species (Fig. 4), suggesting that it may be a dimer or higher oligomer in solution. The trailing peak eluted at the position of vitamin B<sub>12</sub> (350 Da) and did not contain polypeptides that were visible in SDS-polyacrylamide gels (results not shown).

**Assembly of Ad12 penton base and Ad2 fiber.** The noncovalent complex between the penton base and fiber can form spontaneously when purified subunits are mixed in vitro (6). Complementation between temperature-sensitive mutants of Ad7 and Ad5 indicated that fibers from Ad7 can assemble on penton bases from Ad5 (23), raising the possibility that the fiber binding site on the penton base may be conserved among many adenovirus serotypes. Nondenaturing polyacrylamide gel electrophoresis was used to look for complexes of Ad2 fiber and recombinant penton base proteins. The Ad12 penton base was insoluble in *E. coli* and was recovered from inclusion bodies by dissolution in 6 M urea. Most of the protein precipitated during the removal of denaturant by dialysis, and the soluble fraction migrated as a diffuse band (Fig. 5, lane 2). In contrast, the Ad2 penton base was recovered with high yields by following the identical protocol, and it migrated as a sharp band (Fig. 5, lane 5). When an excess of purified Ad2 fiber (Fig. 5, lane 4) was mixed with samples of refolded preparations of Ad12 or Ad2 penton base, both penton base

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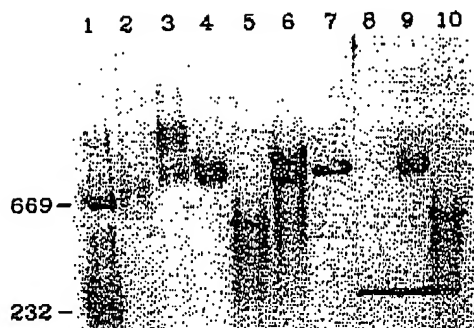


FIG. 5. Electrophoresis of native and recombinant pentons on nondenaturing acrylamide gels. Samples of recombinant Ad12 and Ad2 penton base preparations (lanes 2 and 5) and native Ad2 fiber (lane 4) were mixed and loaded (lanes 3 and 6). Native Ad2 pentons (lane 7) were run for comparison. Lanes 8 to 10, controls showing the specificity of the penton base-fiber assembly. No complexes were detected when Ad2 hexon (lane 8) was mixed with either fiber (lane 9) or recombinant Ad2 penton base (lane 10). The molecular masses (in kilodaltons) of protein standards (lane 1) are indicated.

proteins were quantitatively shifted to species with slower mobilities (lanes 3 and 6). Native penton base-fiber complexes purified from Ad2-infected cells migrated slightly faster than did the complexes formed in vitro from fibers and recombinant Ad2 penton base (Fig. 5; compare lanes 6 and 7). No complexes were detected when a preparation of Ad2 hexon (Fig. 5, lane 8) was mixed with either Ad2 fiber (lane 9) or recombinant Ad2 penton base (lane 10), demonstrating the specificity of the assembly between the penton base and fiber.

**Comparison of the cell adhesion activities of the recombinant Ad2 and Ad12 penton base proteins.** In initial studies, both the Ad2 and Ad12 penton base proteins were found to promote the attachment of adherent cell lines such as A549; however, only the Ad2 protein promoted the adhesion of HeLa S3 suspension cells or caused the rounding of adherent cells at protein concentrations of up to 10  $\mu$ g/ml, suggesting that the RGD sequences in the Ad2 and Ad12 proteins are not functionally equivalent. To extend this analysis, the inhibition of A549 cell attachment to the Ad2 and Ad12 penton base proteins by a panel of antibody and peptide reagents was studied. None of the reagents tested inhibited cell adhesion to polystyrene wells coated with saturating levels of either protein, but significant inhibition by some reagents was observed when subsaturating levels of protein were used to coat the wells. Under these conditions, the patterns of inhibition observed with the Ad2 and Ad12 penton base coatings were not identical. At relatively high densities of Ad2 penton base coating (Fig. 6, solid bars), only two reagents gave a substantial level (>50%) of inhibition, P1F6, a monoclonal antibody specific for the  $\alpha$ v $\beta$ 5-integrin, and the RGD-containing central fragment derived from the wild-type Ad2 penton base (residues 290 to 390). On wells coated with fivefold-less Ad2 penton base (Fig. 6, hatched bars), these two reagents completely blocked cell attachment and the synthetic peptide GRGDSP also reduced cell binding nearly to background levels. Surprisingly, the negative control peptide, GRGESF, also was an effective inhibitor and reduced cell attachment by about 30%. In contrast, the GRGDSP peptide was the most effective inhibitor of A549 cell attachment to the Ad12 penton base and completely blocked cell attachment at coating densities at which the anti- $\alpha$ v $\beta$ 5 monoclonal antibody and the Ad2

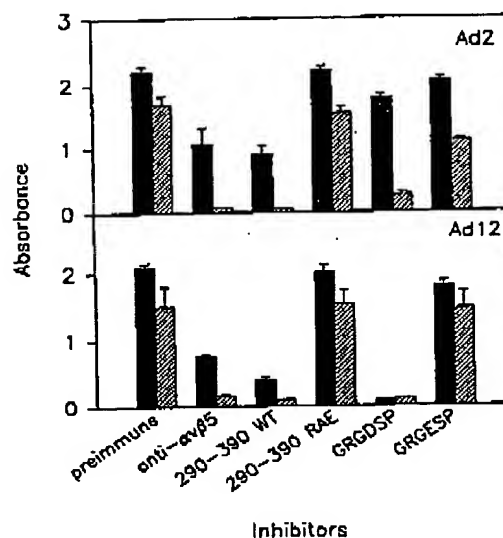


FIG. 6. Inhibition of A549 cell adhesion to Ad2 and Ad12 penton base-coated wells. Suspensions of A549 cells (30  $\mu$ l) were treated for 1 h on ice with 20  $\mu$ l of purified immunoglobulin G from normal rabbit serum (preimmune), with 3  $\mu$ l of anti- $\alpha$ v $\beta$ 5 antibody, with 10  $\mu$ l of the central fragment derived either from the wild-type Ad2 penton base protein (290-390 WT) or from a mutant in which the RGD sequence was changed to RAE (290-390 RAE), or with 10  $\mu$ l of the synthetic peptide GRGDSP or GRGESF. Cells were then diluted with medium and plated in the wells of a 96-well plate that were coated with the penton base protein of Ad2 or Ad12. Data are from wells coated with the minimum amount of protein required for maximal cell adhesion (hatched bars) or with a fivefold-greater protein concentration (solid bars).

central fragment were only partially effective (Fig. 6, solid bars). All three reagents completely blocked cell attachment to a less dense coating of the Ad12 protein (hatched bars); however, the control peptide GRGESF did not substantially inhibit the adhesion of cells to the Ad12 substrate at either density.

A possible explanation for the failure of HeLa S3 cells to adhere to the Ad12 penton base might be the absence of an integrin on these cells that can bind to the Ad12 RGD sequence. Assuming that expression of a specific integrin might be required for efficient infection by Ad12, as appears to be the case for Ad2 (38), then HeLa S3 cells might be relatively more resistant than A549 cells to infection by Ad12. To test this hypothesis, HeLa S3 and A549 cells were exposed to Ad12 virus under identical conditions and the number of infected cells was determined 2 days later by indirect immunofluorescence. As shown in Table 1, HeLa S3 and A549 cells were equally susceptible to infection by Ad12 over a 10-fold range of virus concentration.

**Effect of vitronectin receptor antibodies on infection by Ad12 and Ad2.** The differences in the inhibition of cell binding to the recombinant Ad2 and Ad12 penton bases seen in Fig. 6 suggested that the corresponding viruses might interact differently with integrins in the host cell membrane. To investigate this, cells were treated with monoclonal antibodies specific for integrins  $\alpha$ v $\beta$ 3 and  $\alpha$ v $\beta$ 5 (LM609 and P1F6, respectively) or with the RGD-containing central fragment of the Ad2 penton base (residues 290 to 390) prior to infection by Ad12, Ad2, or Ad2RAE. The number of infected cells was determined 2 days



TABLE 1. Titration of Ad12 virus on HeLa S3 and A549 cells

Virus dilution	No. of Ad12-infected cells per well*	
	HeLa S3	A549
1:1	248 ± 49	227 ± 43
1:3	72 ± 21	70 ± 15
1:9	28 ± 10	25 ± 6

\* Data are means of infected cells in quadruplicate wells ± standard deviations.

later by indirect immunoassay. All three reagents inhibited the infection of both HeLa and A549 cells by Ad12 but had no effect on infection by wild-type Ad2 or the mutant Ad2RAE (Fig. 7). Increasing doses of P1F6 failed to inhibit Ad12 infection completely and markedly enhanced the efficiency of infection of A549 cells by Ad2 (Fig. 8). The magnitude of enhancement of Ad2 and Ad2RAE infection by P1F6 (and by the Ad2 penton base central fragment [results not shown]) on both cell lines varied within the range of 100 to 175% relative to that of untreated controls. Similarly, the level of inhibition of Ad12 infection also varied but never exceeded the levels shown in Fig. 8 for HeLa cells (75% inhibition). Inhibition of Ad2 and Ad2RAE infection by either P1F6 or the Ad2 penton base central fragment was never observed. LM609 was not studied extensively.

Ad2 and Ad12 share fiber receptors. The observation that integrin-blocking reagents inhibit the infection of HeLa and A549 cells by Ad12 but not by Ad2 suggested that Ad2 and Ad12 may bind to different fiber receptors in the host cell plasma membrane. As shown in Fig. 9, this does not appear to be the case. The binding of [<sup>3</sup>H]thymidine-labeled Ad2, Ad2RAE, and Ad12 virions to A549 cells (or HeLa cells [data not shown]) was blocked almost completely by prior treatment

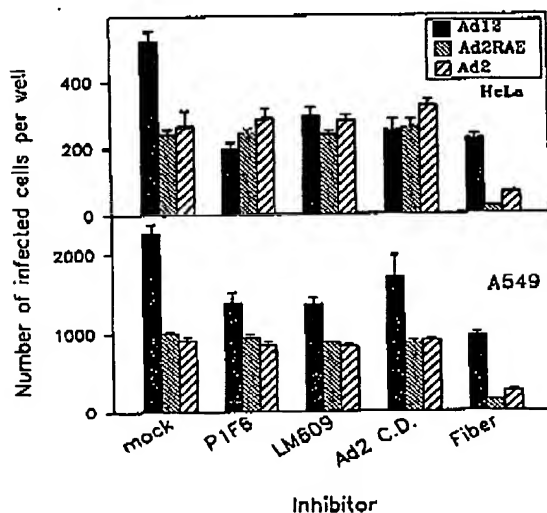


FIG. 7. Effect of receptor-blocking reagents on infection by Ad12, Ad2, and Ad2RAE. HeLa cells and A549 cells were treated in suspension with monoclonal antibodies specific for integrins  $\alpha\beta 5$  and  $\alpha\beta 3$  (P1F6 and LM609, respectively), with the Ad2 penton base central fragment (Ad2 C.D.), or with Ad2 fiber protein (fiber) prior to infection. Data are means of infected cells and standard deviations ( $n = 3$ ).

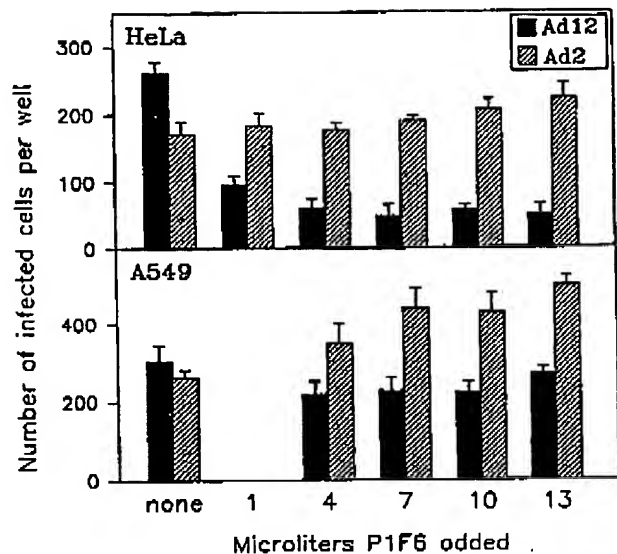


FIG. 8. Effect of increasing doses of P1F6 on infection by Ad2 and Ad12. HeLa cells and A549 cells were treated in suspension with the indicated amounts of P1F6 (anti- $\alpha\beta 5$  monoclonal antibody) ascites fluid prior to infection. Data are means of infected cells and standard deviations ( $n = 3$ ).

of the cells with saturating amounts of purified unlabeled Ad2 virions (data not shown) or Ad2 fiber protein. P1F6 did not inhibit binding, nor did the Ad2 penton base central fragment (data not shown), indicating that vitronectin receptors do not mediate the bulk of virus adsorption. Although the Ad2 fiber protein inhibited the majority of virus binding, it was a relatively weak inhibitor of Ad12 infection. In the experiment shown in Fig. 7, Ad2 fiber protein treatment of HeLa and A549 cells inhibited Ad12 infection by only 57%, whereas Ad2 and Ad2RAE were inhibited by 75% and 88%, respectively.

## DISCUSSION

Both the sequence alignment (Fig. 2) and the penton assembly data (Fig. 5) indicate that the overall structure of the

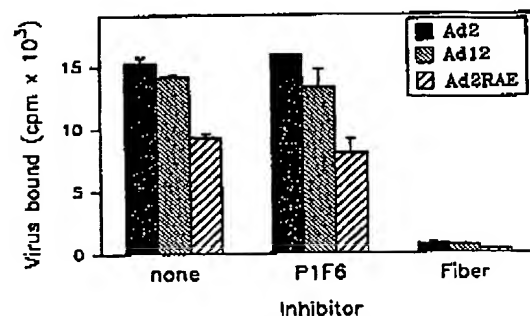


FIG. 9. Effect of P1F6 and Ad2 fiber protein on the binding of Ad12, Ad2, and Ad2RAE to A549 cells. A549 cells were treated in suspension with P1F6 or Ad2 fiber protein prior to infection with  $10^3$  [<sup>3</sup>H]thymidine-labeled virions per cell. After 1 h, cell-associated radioactivity was determined by liquid scintillation counting. Data are the means and standard deviations ( $n = 3$ ).

penton base is conserved among many adenovirus serotypes and suggest a structural model for the protein consisting of two conserved terminal domains joined by a variable-length linker region. It seems likely then that the 63 extra residues in the linker region of the Ad2 protein would be accommodated as a structurally independent  $\Omega$ -like insertion on the protein surface. Evidence that the RGD sequence contained within the linker region is exposed on the external surface of the capsid was presented. Cells saturated with purified Ad2 fiber protein were significantly more susceptible to infection by wild-type Ad2 virus than by the mutant Ad2RAE (Fig. 7), suggesting that the virion RGD sequence can bind directly to integrins and that this interaction can lead to productive infection. The proposed  $\Omega$ -like structure may correspond to the spike on the penton base that was observed by cryoelectron microscopy (30) and, therefore, may be absent or shorter in Ad12, Ad40 and FAV-10. The RGD sequence occurs in the middle of the Ad2 linker region and, therefore, would be located at the distal end of the spike. Constraint of the RGD conformation by this structure might increase its affinity for integrins, as was reported for cyclic RGD peptides (2). High-affinity binding probably is necessary for the rounding and detachment of adherent cells and for the adhesion of suspension cells, two activities of the Ad2 penton base that we could not detect with recombinant Ad12 protein. Alternatively, localization of the RGD sequence at the distal end of a spike might permit the ligand to interact with receptors that, for steric reasons, are not accessible to the Ad12 penton base. The observation that vitronectin receptor antibodies inhibit infection by Ad12 but not by Ad2 (Fig. 7) supports the hypothesis that these integrins do not interact with Ad2. This hypothesis is also supported by the observation that although the Ad2 fiber protein blocks the adsorption of the bulk of Ad12 virions (Fig. 9), it is a weak inhibitor of Ad12 infection (Fig. 7), which indicates that an alternate receptor can mediate Ad12 infection and that this infectious pathway is not efficiently used by Ad2. The observation that Ad2 fiber is a more effective inhibitor of infection by Ad2RAE than by wild-type Ad2 (Fig. 7) supports the conclusion that the alternate or fiber-independent pathway of infection depends on the viral RGD sequence and, therefore, is probably mediated by one or more receptor species in the integrin family.

In most of our experiments, the treatment of cells with vitronectin receptor antibody P1F6 (specific for the  $\alpha\beta 5$ -integrin) enhanced infection by Ad2 and Ad2RAE but inhibited infection by Ad12 (Fig. 8). Similar effects were observed with the Ad2 penton base central fragment (data not shown). These data suggest that both Ad2 and Ad12 have an interaction with vitronectin receptors, but the consequences of this interaction may be different. Further evidence that vitronectin receptors perform a necessary function in the major pathway of Ad2 infection was the observation that mutant cells which lack functional vitronectin receptors were refractory to infection by Ad2 (38). The fiber inhibition data strongly suggest that the vitronectin receptor alone is sufficient for infection by Ad12, but this pathway is not efficiently used by Ad2. Our earlier report that Ad2 mutants which lack functional penton base RGD ligands are as infectious as wild-type Ad2 (3) also supports the conclusion that the vitronectin receptor alone is not sufficient for Ad2 infection. The observation that infection by the Ad2 RGD mutants was delayed on adherent cells but not on suspension cells (3) suggests that cells can constitutively provide the vitronectin receptor function that is necessary for efficient infection by Ad2 and that in the absence of the penton base RGD sequence, this function is performed at a rate that may depend on the density of available vitronectin receptors in

the cell membrane. Since many of the vitronectin receptors on adherent cells are sequestered in focal adhesions, the density of the integrins on these cells available to function in the Ad2 infectious pathway is likely to be lower than that on nonadherent suspension cells. Thus, the Ad2 penton base RGD probably functions to accelerate the rate at which integrins are recruited into the infectious pathway; the magnitude of this acceleration may be considerable on adherent cells but small on suspension cells.

If the vitronectin receptor alone is not sufficient for infection by Ad2, then it may in some way enable the fiber receptor to internalize adsorbed virions. The fact that all the reagents which enhanced Ad2 infection were multivalent with respect to integrin binding (Fig. 4 provides evidence that the Ad2 penton base central fragment is oligomeric) and that each virion fiber is surrounded by a pentameric array of penton base RGD sequences suggests that cross-linking of vitronectin receptors in the vicinity of the fiber receptor may be required for efficient Ad2 infection. Cross-linking vitronectin receptors may transduce signals that ultimately enable the endocytosis of the fiber receptor. Our earlier finding that the infection of adherent cells by Ad2 RGD mutants was not accelerated by coinfection with wild-type Ad2 (3) suggests that the relevant effects of the proposed integrin-mediated signal transduction might be localized to the site of virion attachment. As a precedent, the attachment and entry of several pathogenic enteric bacteria are accompanied by localized changes in the host cell membrane that result from integrin-mediated signal transduction events (5), although the mechanism of integrin signalling in these cases has not been described. Studies employing monovalent antibody fragments or RGD peptides should provide further insight into the role of integrins in infection by Ad2.

If the integrin cross-linking hypothesis is correct and the same fiber receptor species mediates adsorption of both Ad2 and Ad12, then the failure of vitronectin receptor antibodies to enhance Ad12 infection must be explained. This could be related to possible differences in the rate of dissociation of virus from the fiber receptor. The binding of Ad2 virions to the fiber receptor is essentially irreversible (28), but the dissociation rate of Ad12 virions from cells has not been studied. Since Fig. 9 shows that the bulk of Ad2 and Ad12 virion binding is fiber dependent, then efficient use of the alternate, fiber-independent pathway of infection may require the reversible binding of virions to the fiber receptor. The inefficient use of this pathway by Ad2 may reflect the slow dissociation rate of Ad2 fibers from the fiber receptor. Conversely, the efficient use of this pathway by Ad12 implies a faster dissociation rate of Ad12 fiber from its receptor. Vitronectin receptor antibodies may both enhance the uptake of Ad12-fiber receptor complexes and inhibit the fiber-independent pathway; the net result may reflect relative receptor densities and/or other aspects of host cell physiology that may contribute to the variations in the magnitude of enhancement or inhibition of infection that we observed. These same factors, in addition to the differences in host cell types, antibody epitope specificities, and amounts of antibody used could account for the discrepancies between our results and those reported by others that showed the inhibition of Ad2 infection by vitronectin receptor antibodies (4, 38).

Another study showed that purified fibers or virions from Ad2 did not efficiently block the infection of A549 or KB cells by Ad3, a distant relative from virus subgroup B (9). Given the present example that distantly related adenovirus serotypes may bind to the same fiber receptor but that the events subsequent to binding may determine which of several alternate entry pathways may be taken, then infectivity inhibition



assays may not reliably indicate whether different serotypes share fiber receptors. It will be interesting to determine whether all adenoviruses that share the Ad2 fiber receptor also share a requirement for vitronectin receptors. A cell-rounding and -detaching activity was found in an earlier study (35) to be associated with purified pentons of several adenovirus serotypes, including 3 and 11 (subgroup B) and 1, 2, 5, and 6 (subgroup C), which suggests that the RGD sequence and consequent interaction with integrins may be common to these serotypes and that they might share fiber receptors. What are the advantages of this double receptor mechanism for virus infection? Accelerated entry of virions resulting from integrin-mediated signal transduction events would minimize the exposure of adsorbed virions to antibodies or other host defenses that act extracellularly. In addition, adenovirus serotypes such as Ad12 that can use alternate pathways of cell entry may have a relatively broad host cell range.

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